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the FEBS Journal

YidC is required for the assembly of the MscL homopentameric pore

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Keywords
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The mechanosensitive channel with large conductance (MscL) of *Escherichia coli* is formed by a homopentameric assembly of MscL proteins. Here, we describe MscL biogenesis as determined using *in vivo* approaches. Evidence is presented that MscL is targeted to the inner membrane via the signal recognition particle (SRP) pathway, and is inserted into the lipid bilayer independently of the Sec machinery. This is consistent with published data. Surprisingly, and in conflict with earlier data, YidC is not critical for membrane insertion of MscL. In the absence of YidC, assembly of the homopentameric MscL complex was strongly reduced, suggesting a late role for YidC in the biogenesis of MscL. The data are consistent with the view that YidC functions as a membrane-based chaperone 'module' to facilitate assembly of a subset of protein complexes in the inner membrane of *E. coli*.

Introduction

Membrane proteins are responsible for a variety of cellular functions, such as solute transport, protein trafficking, energy transduction and cell division. Similar to soluble proteins, most membrane proteins function in oligomeric complexes. The integral inner membrane proteins (IMPs) of Gram-negative bacteria such as *Escherichia coli* require several distinct targeting and insertion pathways to reach their final destination in the inner membrane [1]. However, the exact requirements for targeting and membrane insertion have been tested for only a few model IMPs. From these studies, a picture has emerged in which targeting and insertion 'modules' (proteins or protein complexes) connect to form a pathway for biogenesis of a specific IMP [2].

The majority of the limited subset of IMPs studied to date insert co-translationally into the inner membrane. At an early stage in synthesis, the ribosome–nascent chain complex is targeted to the membrane via the signal recognition particle (SRP) and its receptor FtsY, which connect the complex to the general Sec translocon in the inner membrane [3]. The Sec translocon is a membrane-integrated machinery, which translocates unfolded polypeptides across and inserts hydrophobic sequences of IMPs into the inner membrane. The core of the translocation machinery

Abbreviations
AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid disodium salt; DDM, *n*-dodecyl-β-D-maltopyranoside; Ffh, fifty four homologue; IMP, inner membrane protein; IMV, inverted membrane vesicle; IPTG, isopropyl thio-β-D-galactoside; SCAM, substituted cysteine accessibility method; SRP, signal recognition particle.

consists of the integral membrane proteins SecY and SecE and the peripheral ATPase SecA [4]. YidC [1,5,6] acts as a Sec-associated protein during insertion of IMPs, probably by facilitating partitioning of hydrophobic transmembrane segments from the Sec translocon into the lipid bilayer. YidC has also been implicated in the folding and quality control of IMPs. The central and versatile role of the YidC 'module' in IMP biogenesis is further exemplified by its function as a Sec-independent insertase for a subset of small IMPs or IMP domains that may reach YidC via the SRP or via direct connection with the translating ribosome.

The substrate specificities of the dedicated IMP targeting and insertion modules SRP/FtsY and YidC are still unclear, which may in part be due to the limited subset of IMPs analysed. Also, little is known about the exact function(s) and mode of action of YidC. Structural analysis of YidC has so far been limited to the non-essential periplasmic domain of YidC [7,8]. YidC is an essential protein in *E. coli*, and YidC depletion in a conditional mutant was found to have a profound effect on the biogenesis of respiratory chain complexes. In particular, the c subunit of F_1F_0 ATP synthase (F_0c) and the N-terminal part of subunit a of cytochrome *o* oxidase have been shown to insert via YidC, independently of the Sec translocon, indicating a requirement for YidC in biogenesis of these hetero-oligomeric complexes (reviewed in [5]). In a similar fashion, the yeast mitochondrial Oxa1 protein, which is homologous to YidC, functions as an essential membrane insertase for subunits of cytochrome bc_1 oxidase and ATP synthase complexes [9].

In this study, we have analysed the biogenesis of MscL using *in vivo* insertion and assembly assays. MscL is an IMP that assembles into a homopentameric complex in the *E. coli* inner membrane to form a gated pore that permits solute efflux upon osmotic downshift [10]. MscL is a suitable model protein to study various aspects of membrane protein biogenesis because it is small and, after membrane insertion, assembles into a pentameric complex for which the structure is known [11,12]. This allows analysis of targeting and membrane insertion of the monomer, as well as complex assembly and quality control. Information about these late steps in IMP biogenesis is very scarce. Using mutants compromised for SRP, Sec or YidC functioning, we found that the SRP is required for optimal targeting of MscL but the Sec translocon is not needed for insertion, consistent with published data [13]. However, in conflict with earlier data [13], depletion of YidC had no major effect on the insertion of MscL, but formation of the pentamer was almost completely abolished under these conditions, suggesting a novel role for YidC in assembly of the MscL complex.

Results

MscL requires SRP for efficient targeting to the inner membrane, but neither SecE nor YidC are critical for insertion of MscL

We investigated the targeting, membrane insertion and oligomeric assembly of the IMP MscL, which spans the membrane twice with an 'N-in, C-in' topology (Fig. 1). To be able to regulate the expression of MscL in various genetic backgrounds, its coding sequence was cloned into several expression vectors. In addition, a haemagglutinin (HA) tag was fused to the C-terminus to allow immunodetection.

We initially explored protease mapping as a method to analyse membrane insertion of MscL. Cells expressing MscL–HA were pulse-labelled, converted to spheroplasts and treated with proteinase K to degrade the external (periplasmic) protein domains. However, MscL was not cleaved under these conditions, in contrast to known periplasmic control proteins, indicating that the small periplasmic domain is not accessible and/or susceptible to the protease (data not shown).

In an alternative strategy to monitor membrane insertion of MscL, we used a substituted cysteine accessibility method (SCAM), using the membrane-impermeable sulfhydryl reagent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid disodium salt (AMS) [14–16]. A unique cysteine was introduced into the periplasmic loop of MscL at position 54 (MscL F54C). Based on the structure of the *Mycobacterium tuberculosis* MscL homologue, this position is expected to be exposed and relatively distant from the membrane, and should therefore be accessible to externally

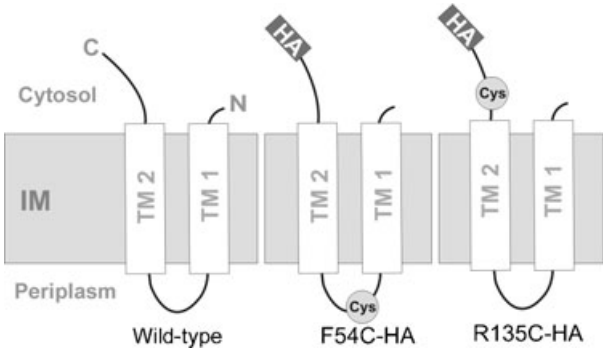

Fig. 1. Schematic representation of the membrane topology for the MscL derivatives used in this study.

added AMS [11] (Fig. 1). As a negative control, we constructed the MscL R135C mutant, which has a single cysteine residue at the C-terminus of the protein (Fig. 1). After membrane insertion, the residue is located in the cytoplasm and should be inaccessible to externally added AMS. The introduced substitutions did not interfere with MscL functioning, suggesting that membrane targeting, insertion and oligomerization of MscL were not affected (data not shown).

To analyse the accessibility of the cysteines, MscL expression was induced, followed by pulse labelling with [^{35}S]methionine. After 2 min, cold methionine was added to stop the labelling, and cells were collected and incubated for 10 min in buffer containing EDTA. This treatment permeabilizes the outer membrane to facilitate access of AMS, which was added subsequently. After 5 min of incubation, unbound AMS was quenched with β-mercaptoethanol, and the samples were subjected to immunoprecipitation using anti-HA serum followed by SDS–PAGE and phosphorimaging. Derivatization of MscL using AMS was detected by a small shift in mobility in SDS–PAGE due to the added molecular mass of AMS (0.5 kDa). In control samples, cells were lysed prior to AMS treatment to allow access to cysteines exposed in the cytoplasm.

First we used SCAM to analyse the role of YidC in membrane insertion of MscL. The MscL derivatives were expressed in strain FTL10 carrying the *yidC* gene under the control of an arabinose-inducible promoter [17]. In both the presence and absence of arabinose, MscL F54C was efficiently derivatized with AMS, suggesting that, irrespective of the presence of YidC, most of the MscL produced during pulse labelling is inserted into the inner membrane, with its periplasmic loop properly located in the periplasm (Fig. 2A). Upon lysis of the cells expressing MscL F54C, AMS labelling appeared to be even more efficient, suggesting that a very small proportion of MscL F54C is either not inserted or not inserted properly, despite the presence of YidC. The negative control MscL R135C (Fig. 1) was not derivatized under the conditions used unless the cells were disrupted prior to AMS labelling (Fig. 2B). This result shows that AMS does not traverse the inner membrane, thus validating the assay conditions. Western blot analysis of samples taken prior to the pulse labelling confirmed the depletion of YidC.

To evaluate the role of the SecYEG translocon, SCAM was performed in the SecE depletion strain CM124, in which the essential *secE* gene is under the control of an arabinose-inducible promoter. Depletion of SecE results in rapid loss of the complete SecYE core of the translocon [18]. As shown in Fig. 3A,

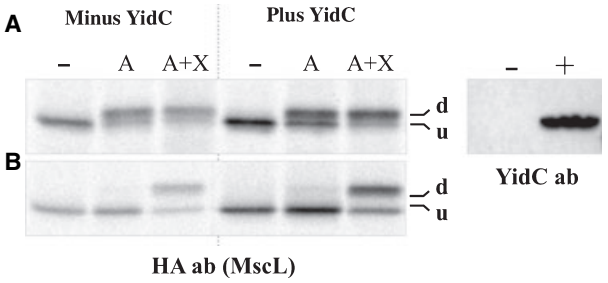

Fig. 2. Membrane insertion of MscL is not significantly affected by depletion of YidC. The single-cysteine mutants of MscL were expressed from the pEH3 vector in the SRP depletion strain FTL10 in the presence or absence of L-arabinose to control the expression of *yidC*. Cells were pulse-labelled with [^{35}S]methionine, and insertion of MscL derivatives was assayed by derivatization of available cysteines using the membrane-impermeable AMS probe, followed by immunoprecipitation using anti-HA serum, SDS–PAGE and phosphorimaging (see Experimental procedures). As a control for the overall accessibility of the cysteines, cells were lysed with a toluene/deoxycholate mixture prior to AMS treatment. (A) MscL F54C and (B) MscL R135C expressed in the absence or presence of L-arabinose (minus/plus YidC). –, mock treatment; A, AMS treatment; A+X, AMS treatment after cell disruption. The panel on the right shows the YidC level in the FTL10 (MscL F54C) cells grown in the absence (–) or presence (+) of L-arabinose as detected by western blotting using anti-YidC serum. d, derivatized MscL; u, underivatized MscL.

depletion of SecE had no major effect on the derivatization of MscL F54C, suggesting that insertion of MscL into the inner membrane occurs independently of the Sec translocon. SecE depletion was verified by western blotting (Fig. 3A). In addition, inhibition of processing of Sec-dependent pro-OmpA confirmed that the Sec translocon had been efficiently inactivated in the SecE-depleted cells (Fig. 3A).

The SRP is the only targeting factor known in *E. coli* that specifically targets membrane proteins to the insertion site in the inner membrane. As defective targeting obstructs membrane insertion, the role of the SRP could be investigated by SCAM using strain FF283, which carries the 4.5S RNA gene encoding the essential RNA component of the SRP under control of the *lac* promoter [19]. As shown in Fig. 3B, depletion of 4.5S RNA significantly inhibited AMS derivatization of MscL. Lysis of the cells prior to AMS treatment restored derivatization, indicating that part of the MscL remains cytosolic upon depletion of SRP. Depletion of 4.5S RNA is known to compromise SRP-mediated targeting, partly because fifty four homologue (Ffh) is unstable in the absence of 4.5S RNA (Fig. 3B) [20]. Inhibition of processing of the SRP-dependent protein CyoA in cells grown under identical conditions confirmed the depletion of functional SRP (Fig. 3B).

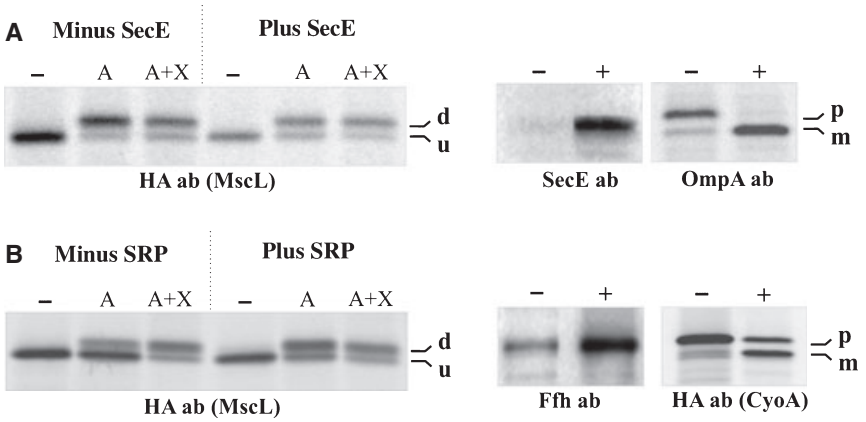

Fig. 3. Membrane insertion of MscL is dependent on prior targeting via the SRP, but does not require the Sec translocon. (A) MscL F54C was expressed from the pEH1 vector in the SecE depletion strain CM124 in the presence or absence of L-arabinose to control the expression of *secE*. Cells were pulse-labelled with [^{35}S]methionine, and insertion of MscL F54C was assayed by derivatization of the cysteine using the membrane-impermeable AMS probe as described in Fig. 2. The middle panel shows a western blot analysis of whole-cell samples using anti-SecE serum to confirm physical depletion of SecE. The panel on the right shows western blot analysis of whole-cell samples using anti-OmpA serum to confirm functional SecE depletion in CM124 cells grown in the absence (–) of L-arabinose by inhibition of processing of pro-OmpA (p) into mature (m) OmpA, compared to cells grown in the presence (+) of L-arabinose. (B) MscL F54C was expressed from the pASK-IBA3c vector in the 4.5S RNA depletion strain FF283 in the presence or absence of IPTG to control the expression of 4.5S RNA. Cells were pulse-labelled with [^{35}S]methionine, and insertion of MscL F54C was assayed by derivatization of the cysteine with the membrane-impermeable AMS probe as described in Fig. 2. The middle panel shows a western blot of whole-cell samples using anti-Ffh serum to show the reduced levels of Ffh upon 4.5S RNA depletion. The panel on the right shows western blot analysis of whole-cell samples of parallel FF283 cultures expressing CyoA–HA from pASK-IBA3 plasmid using anti-HA serum to confirm compromised SRP-mediated targeting in the FF283 cells grown in the absence (–) of IPTG by inhibition of processing of pre-CyoA–HA (p) into mature (m) CyoA–HA as compared to cells grown in the presence (+) of IPTG.

In an independent approach to evaluate the requirements for membrane insertion of MscL, we analysed the MscL content of purified inner membranes from cells compromised in expression of SRP, YidC or the Sec translocon. Cells of strains FTL10, CM124 and FF283 harbouring an MscL–HA expression plasmid were grown to early log phase in the presence of inducers that sustain expression of YidC, SecE and 4.5S RNA, respectively. The cells were washed and resuspended in medium with (positive control) or without inducers to deplete YidC, SecE or 4.5S RNA. After continued growth and depletion, expression of MscL–HA was induced for 1 h. The cells were collected and inner membrane vesicles (IMVs) were prepared via isopycnic sucrose gradient centrifugation. IMV samples were normalized based on protein content, and analysed by SDS–PAGE and western blotting. As shown in Fig. 4A (left panels), depletion of YidC or SecE did not result in significant reduction of the amount of MscL–HA that co-purified with the inner membranes. To confirm that the co-purified MscL–HA is inserted as an integral membrane protein, rather than being peripherally attached, the IMVs were extracted with sodium carbonate to remove peripheral membrane proteins. Irrespective of the depletion of YidC or SecE, MscL–HA could not be extracted from the membrane preparations, indicating that the protein is fully integrated into the lipid bilayer (Fig. 4A, right panels). This corroborates our results from the SCAM assay, and again suggests that neither YidC nor SecE is critical for membrane insertion of MscL. In contrast, upon depletion of 4.5S RNA, the MscL–HA content of the IMVs was clearly reduced, consistent with the AMS derivatization data, suggesting a pivotal role for the SRP in MscL targeting (Fig. 4A, left panels). As a control for the carbonate extraction procedure, we verified that the cytosolic phage shock protein A (PspA), which is upregulated upon YidC depletion [21] and to some degree co-purifies with the IMVs [22], is extracted by the carbonate treatment. In contrast, YidC, which is itself an integral inner membrane protein, was resistant to the extraction, as expected (Fig. 4B).

Depletion of YidC (but not SecE) affects oligomeric assembly of MscL in the inner membrane

Upon insertion of MscL into the inner membrane, the monomers must assemble into a pentamer to form a

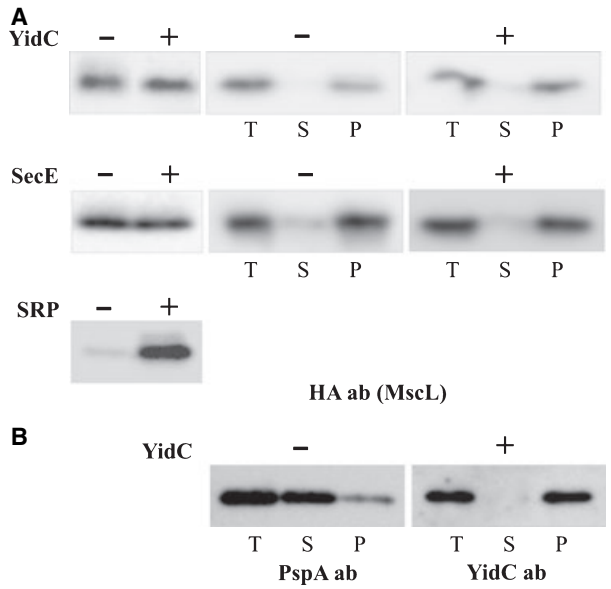

Fig. 4. Depletion of SRP, but not of YidC and SecE, leads to a decreased amount of MscL subunit in the inner membrane. (A) SDS–PAGE and western blot analysis using anti-HA serum to detect MscL subunit levels in IMVs derived from FTL10, CM124 or FF283 cells depleted for YidC, SecE or 4.5S RNA, respectively. Left panels: amount of MscL co-purified with IMVs depleted (–) or not depleted (+) for the indicated factors. Right panels: sodium carbonate extraction of the IMVs to distinguish integral and peripheral membrane proteins. T, total IMV sample; S, carbonate supernatant fraction; P, carbonate pellet fraction. (B) As a control for the carbonate extraction procedure, PspA (a peripheral IMP) and YidC (an integral IMP) were detected in YidC-proficient IMVs by western blotting using anti-PspA and anti-YidC serum, respectively.

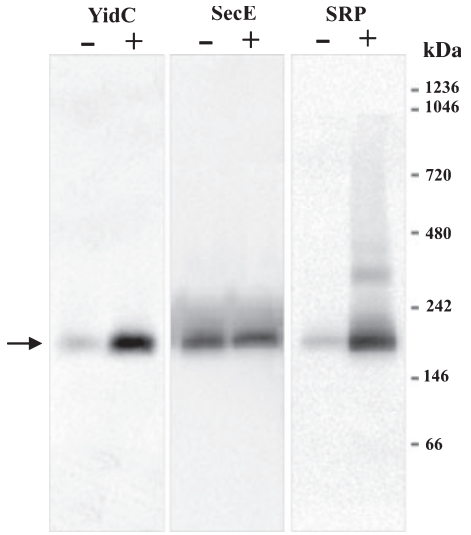

Fig. 5. Formation of the MscL pore complex is strongly dependent on YidC but is not affected by depletion of SecE. Native gel analysis of the IMVs used in Fig. 4, to monitor the effect of YidC, SecE and SRP depletion on the level of the MscL pentamer in the inner membrane. The IMVs were solubilized with DDM, and subjected to BN PAGE and western blotting using anti-HA serum to detect the MscL–HA complex. The calculated molecular mass of the MscL pentamer is 74 kDa. Under native conditions, the MscL complex runs at an apparent molecular mass of ~ 180 kDa (arrow).

functional mechanosensitive channel with large conductance. The molecular mechanism of MscL folding, oligomerization and quality control has remained unexplored. Given recent evidence that, for certain IMPs, YidC is not only required for membrane insertion of individual subunits, but also for assembly of those subunits in higher-order complexes [6,23], we examined the role of YidC in assembly of the MscL complex. To this end, IMVs derived from YidC-depleted cells and control cells expressing MscL–HA (see above) were solubilized using *n*-dodecyl-β-D-maltopyranoside (DDM) and membrane protein complexes were separated by Blue Native PAGE (BN PAGE) and transferred to polyvinylidene fluoride membrane. It should be noted that the IMVs used were identical to the IMVs used in Fig. 4 to show that the total level of MscL is equivalent in the YidC-depleted and control IMVs. The MscL complexes on the polyvinylidene fluoride membrane were detected with HA antibody. In control IMVs, the anti-HA serum reacts with a band at ~ 180 kDa that presumably represents the MscL–HA pentamer. The aberrant electrophoretic mobility is probably due to binding of the detergent (DDM) used for solubilization of the pentameric complex. Notably, MscL expressed at endogenous levels migrates at a similar position during BN PAGE (data not shown), indicating that the MscL–HA complex represents a functional pentamer. Strikingly, in the YidC-depleted IMVs, the MscL complex is hardly detected, although the level of MscL–HA in the membranes is equal to that of the non-depleted IMVs. This indicates that YidC is required for assembly of the MscL complex (Fig. 5).

To investigate the role of the Sec translocon in formation of the MscL–HA complex, SecE-depleted IMVs and control IMVs were analysed by BN PAGE and western blotting. As shown in Fig. 5, depletion of SecE did not have a significant impact on the level of the MscL–HA complex, suggesting that the Sec translocon is dispensable for the oligomerization of the MscL subunits.

Discussion

We have analysed the requirements for targeting, membrane insertion and oligomerization of the MscL

complex in the *E. coli* inner membrane. The homopentameric MscL pore is part of a turgor-responsive solute efflux system that protects bacteria from lysis upon osmotic downshift (reviewed in [24]). Using *in vivo* approaches, we found that formation of the MscL pentamer, but not insertion of the MscL monomer into the inner membrane, strongly depends on YidC. The Sec translocon appears to be dispensable for both MscL insertion and oligomerization, but optimal membrane targeting requires the SRP.

Membrane integration of MscL was investigated by analysing the derivatization of single cysteines engineered in the periplasmic and cytoplasmic loops of MscL, respectively, using the membrane-impermeable AMS reagent. A recent study that appeared during preparation of the current paper used a very similar SCAM approach to study the requirements for targeting and integration of MscL, but the authors used MscL derivatives with cysteines introduced at slightly different positions, i.e. periplasmic mutation I68C and cytoplasmic control S136C [13]. Consistent with our data, efficient integration of MscL was found to occur in the absence of a functional Sec translocon and to be affected by depletion of the SRP, although in the latter case the reported effect was much more pronounced than in the present study. However, the authors reported YidC-dependent integration of MscL into the inner membrane, inferred from the diminished derivatization of the I68C mutant upon depletion of YidC. This contrasts with our finding that depletion of YidC had no effect on the insertion of MscL, when using the F54C mutant. In addition, in our hands, the quantity of MscL present in the inner membrane appeared to be unaltered upon YidC depletion (Fig. 4A, left panel). The reason for this discrepancy is not clear, but might be explained by the structural constraints of the respective mutants used for the assays. The structure of MscL of *E. coli* is unknown, but may be modelled from the crystal structure of the MscL homologue from *Mycobacterium tuberculosis* [11]. In this model, position 54, which was analysed in the present study, appears to be well exposed in the periplasm, with a maximal distance to the plane of the lipid bilayer. In contrast, position 68, which was used in the earlier study [13], is located adjacent to the centre of the pore-forming TM1. It is therefore conceivable that even a slight perturbation of the conformation of MscL, for example due to the absence of YidC, might hinder access of AMS to position 68, thus minimizing derivatization of the MscL subunits. In contrast, accessibility of the more exposed position 54 might be less sensitive to structural alterations.

Our results do imply an important role for YidC in biogenesis of the MscL complex, but not at the level of membrane insertion, as the level of pentameric MscL complex in the inner membrane was strongly reduced upon depletion of YidC. This indicates a late role for YidC in formation of the MscL complex after insertion of the monomer into the membrane (Fig. 5). Corroborating these data, it has been shown recently using an independent proteomic approach that the quantity of complexed MscL (expressed at the endogenous level) was significantly reduced in YidC-depleted inner membranes (D. Wickström, unpublished results). Apparently, in the absence of YidC, the pentameric MscL complex either does not form or is so unstable that it disassembles during BN PAGE. The exact stage and mechanism of YidC functioning in MscL assembly remains unclear. YidC could be required for folding of the MscL monomer into an assembly-competent conformation. Alternatively, YidC could play a more direct role in assembly of the pentameric complex from MscL monomers.

The versatile role of YidC in membrane protein biogenesis in *E. coli* is underscored by *in vitro* studies showing that YidC is critical for folding and stability of the monomeric lactose permease, rather than for its insertion in the membrane [25]. Furthermore, we have shown recently that YidC is involved in assembly of the $MalFGK_2$ maltose transport complex [23]. YidC was not essential for insertion of MalF into the inner membrane, but was essential for its folding and stability, thus affecting the downstream assembly of the $MalFGK_2$ complex [23]. In this respect, it is of interest to note that, in yeast mitochondria, deletion of the *yidC* homologue *oxa1* can be compensated for by simultaneous deletion of *yme1*, which encodes a membrane protease that is responsible for degradation of unassembled subunits of ATP synthase. This indirectly argues that Oxa1 functioning is critical for assembly of the ATP synthase subunits rather than their individual insertion into the membrane [26].

If neither YidC nor the Sec machinery is absolutely required for membrane insertion of MscL subunits, how do MscL subunits partition into the lipid bilayer? In the most likely scenario, MscL can make promiscuous use of the two insertases. Unfortunately, attempts to produce a double SecE and YidC conditional strain to test this supposition have been unsuccessful. Alternatively, it may be possible for MscL to be inserted unassisted, provided that it is delivered to the membrane by the SRP targeting pathway. It is of interest to note that, even in the presence of YidC, full MscL insertion appears to be a slow process [13]. Intriguingly, the osmosensor protein KdpD, which has four closely spaced transmembrane

domains, has been shown to insert independently of the Sec translocase and YidC, similar to MscL [27]. This may be related to the relatively small periplasmic domains present in both proteins, although other IMPs with similar characteristics have been shown to insert via the YidC insertase [6]. Hence, it is likely that specific characteristics of the transmembrane pairs are also critical for the conditions of membrane insertion.

Analysis of the biogenesis of more and more IMPs has revealed many different requirements for targeting, insertion and oligomerization. These findings reinforce the idea that targeting and insertion factors function as modules that may be redundant but can be connected to form a functional biogenesis pathway for a specific IMP [2].

Experimental procedures

Materials

Restriction enzymes, the Expand long-template PCR system and Lumi-Light Plus western blotting substrate were purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). [^{35}S]methionine and Protein A Sepharose were purchased from Amersham Biosciences (Uppsala, Sweden). T4 ligase, alkaline phosphatase and 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid disodium salt (AMS) were purchased from Invitrogen (Carlsbad, CA, USA). Antiserum against influenza haemagglutinin (HA) was obtained from Sigma (St Louis, MO). The other antisera used were from our own collection. For phosphorimaging, a Storm 820 scanner and associated IMAGEQUANT software from Molecular Dynamics (Sunnyvale, CA, USA) were used.

Bacterial strains and growth conditions

Escherichia coli TOP10F strain (Invitrogen) was used for routine cloning and was cultured at 37°C in Luria–Bertani (LB) broth supplemented with 12.5 μg·mL^{-1} tetracycline. The 4.5S RNA depletion strain FF283 [19], the SecE depletion strain CM124 [18] and the YidC depletion strain FTL10 [17] were grown as described previously [17,28]. Expression of the MscL mutants was induced using 1 mM isopropyl thio-β-D-galactoside (IPTG) for the pEH1- and pEH3-derived plasmids [29], with 0.2 μg·mL^{-1} anhydrous tetracycline for the pASK IBA3c-derived plasmids (IBA GmbH, Göttingen, Germany) and with 0.2% L-rhamnose for the pRha67-derived plasmids [30].

Construction of MscL cysteine mutants

MscL was amplified from *E. coli* K12 genomic DNA, including a C-terminal HA tag, using primers 5′-GCGCGCGA ATTCATGAGCATTATTAAAGAATTTCG-3′ (forward) and 5′-CGCGCGGGATCCTTAAGCATAATCAGGAAC ATCATAAGGATAACCACCAGGAGAGCGGTTATTC TGCTCTTTC-3′ (reverse). The *Eco*RI/*Bam*HI-digested PCR fragment (MscL–HA) was cloned into pC4Met [31]. To construct the single-cysteine mutants, the phenylalanine at position 54 or the arginine at position 135 were substituted by cysteine using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA, USA). The mutagenic primers used to construct MscL R135C were 5′-AGCAGAATAA CTGCTCTCCTGGTG-3′ (forward) and 5′-CACCAGGAG AGCAGTTATTCTGCT-3′ (reverse), and those for MscL F54C were 5′-GGGATCGATTGCAAACAGTTTGC-3′ (forward) and 5′-GCAAACTGTTTGCAATCGATCCC-3′ (reverse). Subsequent DNA sequencing confirmed the substitutions at the indicated positions. The new constructs were cloned into the above-mentioned vectors to allow expression in various genetic backgrounds. Functionality of the MscL derivatives was confirmed as described previously [32].

Biochemical assays

For AMS derivatization [14], cells were grown in M9 minimal medium. Expression of MscL derivatives was induced for 3 min by addition of 1 mM IPTG for pEH vectors and 0.2 μg·mL^{-1} anhydrotetracycline for pASK-IBA vectors, followed by pulse labelling with [^{35}S]methionine (30 μCi·mL^{-1}) for 2 min. ^{35}S labelling was stopped by adding an excess (15 mM) of cold methionine, and cells were harvested and resuspended in derivatization buffer (50 mM Hepes pH 7.0, 150 mM NaCl, 2 mM EDTA). The cell suspensions were divided into three aliquots, and 10% toluene and 0.2% sodium deoxycholate were added to one aliquot to disrupt the cells. The aliquots were equilibrated at 30°C for 10 min. Subsequently, 500 μg·mL^{-1} AMS was added to two aliquots (one containing the disrupted cells), followed by continued incubation at 30°C for 5 min. Subsequently, all aliquots were quenched using 10 mM β-mercaptoethanol for 10 min on ice, and subjected to immunoprecipitation using anti-HA serum followed by SDS–PAGE and phosphorimaging. IMVs were prepared essentially as described previously [33]. To distinguish peripheral from integral IMPs, IMVs were extracted with 0.2 M Na_2CO_3 as described previously [31]. Carbonate-insoluble and supernatant fractions were analysed by SDS–PAGE and western blotting. To resolve IMP complexes, IMVs were subjected to BN PAGE using pre-cast 4–16% gradient NativePAGE™ Novex® gels from Invitrogen. Membrane samples were solubilized for 15 min on ice using 0.5% DDM (final concentration). Samples were centrifuged at 100 000 ***g***, and solubilized protein complexes were recovered from the supernatant, mixed with sample buffer, and run using the supplied buffers and reagents according to the manufacturer's protocol (Invitrogen). Resolved protein complexes were blotted onto polyvinylidene fluoride membranes, and MscL–HA complexes were identified by western blotting using anti-HA serum.

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